AD_____

Award Number: DAMD17-01-1-0419

TITLE: Telomere Length and Genomic Stability as Indicators of Breast Cancer Risk

PRINCIPAL INVESTIGATOR: Ying Zou , M.D.

CONTRACTING ORGANIZATION: The University of Texas Southwestern Medical Center at Dallas
Dallas, Texas 75390-9105

REPORT DATE: May 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

# REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188



| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | May 2004 | Annual Summary (1 May 2001 - 30 Apr 2004) |

**4. TITLE AND SUBTITLE**
Telomere Length and Genomic Stability as Indicators of Breast Cancer Risk

**5. FUNDING NUMBERS**
DAMD17-01-1-0419

**6. AUTHOR(S)**
Ying Zou , M.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
The University of Texas Southwestern Medical Center at Dallas
Dallas, Texas 75390-9105

*E-Mail:* ying.zou@utsouthwestern.edu

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**
Original contains color plates: All DTIC reproductions will be in black and white.

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT (Maximum 200 Words)**

Telomeres are repetitive sequences that protect the ends of linear chromosomes and shorten during each cell division. Very short telomeres have been associated with changes in gene expression (in yeast) and decreased genomic stability. We published the first proof that silencing effects can occur at human telomeres. A luciferase reporter near a telomere showed on average a 10-fold reduction in expression relative to internal control genes. The silencing is reversible through inhibition of histone deacetylases and dependent on telomere length. We further demonstrated spontaneously switch on and off of the telomeric genes, resulting in a second publication. We determined that a subset of short telomeres in human genome lead to replicative senescence (aging) and triggered early DNA damage-signaling pathways, resulting in a third publication. We developed and constructed both a 70-mer array and the combination of both fluorescence *in situ* hybridization technique and immunostaining, which can allow us detailed characterization of telomere lengths and specific gene rearrangements that will complement the expression data to create a detailed picture of the behavior of telomeres in the progression of breast cancer.

**14. SUBJECT TERMS**
Telomere/Telomerase, Genome Stability, Subtelomere, Gene Expression, Age-related

**15. NUMBER OF PAGES**
23

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

## Table of Contents

## Introduction

Telomeres are repetitive sequences that protect the ends of linear chromosomes. Telomeres shorten with each cell division; eventually reaching a critically short length that causes the cell to permanently cease dividing after reaching number of divisions known as the Hayflick limit. It has been shown in our lab and others that this telomere initiated growth arrest (termed M1) is mediated through the tumor suppressors p53 and pRb. Inhibiting these proteins with SV40 T antigen or HPV E6/E7 leads to further division until a second limit (M2) is reached. This proliferation in the presence of short telomeres is associated with end to end chromosome fusions and genomic instability [1,2]. This genomic instability occasionally results in the production of a rare clone that can continue to divide due to the activation of telomerase, an enzyme capable of adding new DNA to the telomeres. This situation may parallel the formation of cancerous cells *in vivo*, which in many cases show evidence of genomic rearrangements leading to activation of telomerase [1-4]. End-to-end fusion chromosomes have been shown to have little or no telomeric DNA present at the junction in humans [5] and mice [1]. Note that telomere shortening is correlated with aging, and aging is one of the most important risk factors for sporadic breast cancer. Evidence for telomere length as an important regulator of the intracellular environment beyond its role as a "mitotic clock" originally came from *Drosophila* [6] and has been further characterized in yeast [7]. In Saccharomyces cerevisiae, it has been shown that a transgene integrated near a telomere will be transcriptionally silenced by an active mechanism involving SIR proteins that depends both on the length of the telomere and on the distance to the transgene [8]. More recently, an endogenous gene has been characterized that exhibits this silencing, or telomere position effect (TPE), in yeast [9]. Our work during the first year of this grant extended these observations from yeast to human cancer cells[10]. In the second year, we have further characterized human TPE in human cancer cells[11]. In the third year, we have determined a subset of short telomeres in human genome leading to replicative senescence (aging)[12], resulting in a third publication. We have made significant progress in our efforts to understand the molecular changes that occur in telomeric and subtelomeric regions during breast cancer progression.

# Body

"Task 1" from our statement of work (months 1-12) was to establish the existence of telomere position effect (TPE) in human cancer cells through studies on the behavior of a luciferase reporter gene inserted either at a telomere or at an internal (control) site. As outlined in my previous report, this task was completed ahead of schedule and significantly extended in scope. During the second year, using a fluorescent reporter integrated into human subtelomeres, we have shown that expression switches on and off[11], in contrast to a previous report and in good agreement with the yeast [7] and *Drosophila* [6] model systems. I have also demonstrated using these cells that silencing can be relieved by treatment with 5-bromodeoxyuridine[11].

"Task 2" from my statement of work (months 12-30) was to investigate the effect of transient telomerase expression on the frequency of immortalization of human mammary epithelial cells. This task was completed. An adenoviral vector has used to transiently express telomerase in a population of cells without additional complications since this virus does not integrate into the genome and consequently does not need to be excised. The efficacy of adenoviral infection of these cells was shown using a green fluorescent protein (GFP) vector compared to control cell. Telomerase expression after adenoviral infection was assessed using the telomerase repeat amplification protocol (TRAP) assay and shown to be comparable to the level exhibited by the human tumor cell line H1299. The alteration of average telomere length after adenoviral infection was detected and compared with control cells' by telomere restriction fragment (TRF) assay. The frequency of spontaneous immortalization of human mammary epithelial cells was $5.3 \times 10^{-7}$ determined by fluctuation analysis.

"Task 3" from my statement of work (months 18-36) was to investigate the features of malignant and pre-malignant breast cancer cells by fluorescence in situ hybridization. This task was completed ahead of schedule and significantly extended in scope. A quantitative protocol has been developed for fluorescence *in situ* hybridization (FISH) analysis to telomere repeats [13]. A subset of short telomeres leading to replicative senescence (aging) was determined by FISH analysis using telomere specific probes[12]. By combining FISH analysis using chromosome specific painting probes and immunostaining using early DNA damage response proteins including gamma H2AX[14] and 53BP1[15], we further confirmed that chromosome ends with no telomere repeats trigger DNA damage response and lead to cellular senescence (aging)[12]. Work on characterizing sub-telomeric genes known to be involved in breast cancer, including hTERT has been completed. Normal breast cells have two normal hTERT loci per cell detected by FISH analysis using FITC conjugated telomerase special probes. However, malignant and pre-malignant breast cancer cells have more than two hTERT loci per cell, which suggests that the amplification of telomerase genes to provide limitless replicative potential is required during breast cancer development. In addition to the *in situ* studies, we finished examining the state (presence, absence, or amplification) of subtelomeric genes using a 70-mer array, which generated data on a much larger sample of subtelomeric genes than originally anticipated.

## Key Research Accomplishments

*Original Statement Task 1.* Establish the existence of telomere positive effect in a human cancer cell line through a comparison of luciferase reporter genes inserted either at a telomere or internally, months 1-12;

- We demonstrate the presence of TPE in a human cancer cell. Positive clones containing a luciferase reporter adjacent to a newly formed telomere express 10 times less luciferase expression than do control clones generated by random integration. Luciferase expression is restored by trichostatin A, a histone deacetylase inhibitor. Overexpression of a human telomerase reverse transcriptase complementary DNA results in telomere elongation and an additional 2- to 10-fold decrease in expression in telomeric clones but not control clones.
- We extended our original task 1 to further analyze telomeric clones. Telomeric clones bearing a telomeric reporter were fully characterized and it was demonstrated that expression switches on and off (as opposed to low uniform expression) at telomeres.
- 5-bromodeoxyuridine was found to relieve silencing of both telomeric and internal clones.

*Original Statement Task 2.* Show that elongation of telomeres through transient expression of telomerase leads to a reduction in the frequency of immortalization of human mammary epithelial cells (months 12-30):

- Adenoviral vectors were obtained and shown, using a GFP control vector, to efficiently infect human mammary epithelial cells (HMEs).
- Infection of HMEs with adenoviral hTERT was shown to effectively reconstitute telomerase activity.
- After one unsuccessful experiment, HMEs have now been infected with either hTERT or a control vector and their average telomere lengths were assayed by TRF analysis.
- The frequency of immortalization of human mammary epithelial cells was $5.3 \times 10^{-7}$ determined by fluctuation analysis.

*Original Statement Task 3.* Investigate the features of malignant and pre-malignant breast cancer cells by fluorescence *in situ* hybridization (months 18-30):

- A quantitative protocol has been developed for *in situ* hybridization to telomere repeats.
- A protocol for analysis of the hTERT gene by *in situ* hybridization has been developed and used on immortal and pre-immortal human mammary epithelial cells. We found that normal breast cells have two normal hTERT loci per cell detected by FISH analysis using FITC conjugated telomerase special probes. However, malignant and pre-malignant breast cancer cells have more than two hTERT loci per cell.
- We also extended the original task 3 to further understand the molecular mechanisms of development of beast cancer. A protocol combining FISH analysis using chromosome specific painting probes and immunostaining using early DNA damage response proteins including gamma H2AX[14] and 53BP1[15] has been

developed and used in studying replicative senescence and in the progression of breast cancer. We found that in normal cells the chromosome ends with no telomere repeats co-localized with early DNA damage response, which first demonstrate that telomere signal free chromosome ends trigger DNA damage response, leading to grow arrest and cellular senescence (aging). However, breast cancer cells lost this anti-cancer protection, have an ability to unlimited proliferate and lead to development of breast cancer.

## Reportable Outcomes

Publications

**Baur, J.A., Zou, Y.,** Shay, J.W., and W.E. Wright. Telomere position effect in human cells. Science, 292: 2075-77.

**Baur, J.A.,** Shay, J.W., and W.E. Wright. Spontaneous reactivation of a silent telomeric transgene in a human cell line. Chromosoma, 112: 240-246.

**Zou, Y.,** Shay, J.W., and W.E. Wright. Does a sentinel or a subset of short telomeres determine replicative senescence? (In review)

Abstracts and Presentations

**Baur, J.A., Zou, Y.,** Shay, J.W., and Wright, W. E. Telomere position effect in human cells. Presented at the UT Southwestern GSO Poster Session. November, 2001.

**Baur, J.A., Zou, Y.,** Li, B., de Lange, T., Shay, J.W., Wright, W.E. Characterization of telomere position effect in human cells. Presented at the Molecular Genetics of Aging meeting, Cold Spring Harbor, October, 2002.

**Baur, J.A., Zou, Y.,** Li, B., de Lange, T., Shay, J.W., Wright, W.E. Characterization of telomere position effect in human cells. Presented at the UT Southwestern GSO Poster Session. November, 2002.

**Baur, J.A., Zou, Y.,** Li, B., de Lange, T., Shay, J.W., Wright, W.E. Characterization of telomere position effect in human cells. Presented at The Role of Telomeres and Telomerase in Cancer, San Francisco, December, 2002.

**Zou, Y.,** Shay, J.W., and Wright, W. E. Non-canonical telomeric sequences in the regulation of replicative aging. Presented at The Role of Telomeres and Telomerase in Cancer, San Francisco, December, 2002.

**Zou, Y.,** and W.E. Wright. Non-canonical telomeric sequences in the regulation of replicative aging. Presented at The Role of Telomeres and Telomerase in Cancer, Cold Spring Harbor, 2003.

**Zou, Y.,** Shay, J.W., and Wright, W. E. A subset of short telomeres determine replicative senescence. Presented at the UT Southwestern GSO Poster Session. November, 2003.

Degree

Joseph Baur had completed and obtained his Ph.D. in Aug 2003, which were supported by this award.

A list of personnel receiving pay from the research effort

Ying Zou, M.D. September 2003 – April 2004

Joseph Baur, Ph.D. May, 2001 – August 2003

Cell lines and reagents developed

Hela cell lines containing either an internal or a telomeric luciferase reporter gene have been developed and characterized. Telomeres in several lines were extended through the introduction of telomerase to generate matched pairs having either short or long telomeres next to the reporter gene. A 70-mer array containing most of the sub-telomeric genes in the human genome has been developed. This array includes many genes obtained through collaboration that are not currently in the public genome assembly, and can be used to comprehensively analyze subtelomeric gene status in immortal and pre-immortal human mammary epithelial cells.

## Conclusions

Our work during three years, including experiments outlined for all three original tasks, has resulted in the publication of two papers on human telomere position effect, one paper on short telomere and replicative senescence (aging), and the development of several new techniques. In addition, we were able to present the work at several meetings. The regulation of subtelomeric genes by telomere length could have extremely important implications for breast cancer. Altered expression of subtelomeric genes could indicate the presence of cells with extremely short telomeres that could be expected to be at an increased risk of genomic rearrangements. Increased expression of subtelomeric genes may also have a more active role in the progression of breast cancer. A 70-mer array with most of the sub-telomeric genes in the human genome being developed will prove a valuable tool in the field for assessing the role of subtelomeric rearrangements and misregulation in breast cancer. A subset of short telomeres lead to replicative senescence (aging) determined by *in situ* hybridization techniques and triggered early DNA damage-signaling pathways. The *in situ* hybridization techniques being developed can allow us detailed characterization of telomere lengths and specific gene rearrangements that will complement the expression data to create a detailed picture of the behavior of telomeres in breast cancer formation.

## References


1. Hande, M.P., Samper, E., Lansdorp, P. & Blasco, M.A. Telomere length dynamics and chromosomal instability in cells derived from telomerase null mice. *J Cell Biol* **144**, 589-601. (1999).
2. Blasco, M.A. et al. Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell* **91**, 25-34. (1997).
3. Sawyer, J.R., Husain, M., Pravdenkova, S., Krisht, A. & Al-Mefty, O. A role for telomeric and centromeric instability in the progression of chromosome aberrations in meningioma patients. *Cancer* **88**, 440-53. (2000).
4. Zheng, M.H., Siu, P., Papadimitriou, J.M., Wood, D.J. & Murch, A.R. Telomeric fusion is a major cytogenetic aberration of giant cell tumors of bone. *Pathology* **31**, 373-8. (1999).
5. Wan, T.S. et al. Absence or low number of telomere repeats at junctions of dicentric chromosomes. *Genes Chromosomes Cancer* **24**, 83-6. (1999).
6. Hazelrigg, T., Levis, R. & Rubin, G.M. Transformation of white locus DNA in drosophila: dosage compensation, zeste interaction, and position effects. *Cell* **36**, 469-81. (1984).
7. Gottschling, D.E., Aparicio, O.M., Billington, B.L. & Zakian, V.A. Position effect at S. cerevisiae telomeres: reversible repression of Pol II transcription. *Cell* **63**, 751-62. (1990).
8. Stavenhagen, J.B. & Zakian, V.A. Internal tracts of telomeric DNA act as silencers in Saccharomyces cerevisiae. *Genes Dev* **8**, 1411-22. (1994).
9. Vega-Palas, M.A., Martin-Figueroa, E. & Florencio, F.J. Telomeric silencing of a natural subtelomeric gene. *Mol Gen Genet* **263**, 287-91. (2000).
10. Baur, J.A., Zou, Y., Shay, J.W. & Wright, W.E. Telomere position effect in human cells. *Science* **292**, 2075-7 (2001).
11. Baur, J., Shay, J.W. & Wright, W.E. Spontaneous reactivation of a silent telomeric transgene in a human cell line. *chromosoma* **112**, 240-246 (2004).
12. Zou, Y., Shay, J.W. & Wright, W.E. Does a sentinel or a subset of short telomeres determine replicative senescence? *(in review).*
13. Zou, Y., Yi, X., Wright, W.E. & Shay, J.W. Human telomerase can immortalize Indian muntjac cells. *Experimental Cell Research* **281**, 63-76 (2002).
14. Rogakou, E.P., Pilch, D.R., Orr, A.H., Ivanova, V.S. & Bonner, W.M. DNA double-stranded breaks induce histone H2AX phosphorylation on serine. *J Biol Chem* **273**, 5858-5868 (1998).
15. Rappold, I., Iwabuchi, K., Date, T. & Chen, J. Tumor suppressor p53 binding protein 1 (53BP1) is involoved in DNA damage-signaling pathways. *J Cell Biol* **153**, 613-620 (2001).

inates contamination by microbes, which is an important criterion for maintaining food industry standards. Fermentative growth of naturally heterotrophic microalgae has resulted in dry biomass accumulation to 100 g/liter (*3*, *27*), which is 10 to 50 times the yields obtained by using light-dependent culture systems. Fermentation-based systems can reduce production costs of microalgae by an order of magnitude relative to that incurred by photosynthesis-based production; cost reduction analyses factor in expenses for both fixed-carbon supplementation and equipment operation (*28*). Commercial benefits of fermentation-based systems result from increased biomass, productivity, harvesting efficiency, and reduced losses from contamination. The ability to grow microalgae heterotrophically increases the feasibility for developing a large range of new algal products.

Marine ecosystems also depend on diatoms, which contribute substantially to the reduction of inorganic carbon in marine habitats. Such a contribution may increase substantially as the ecology of oceanic environments is altered (*29*–*32*). The exploitation of diatoms that can be genetically manipulated and that can grow heterotrophically will facilitate the use of mutants to augment our understanding of both photosynthesis and other metabolic pathways that are essential for competing in marine ecosystems.

### References and Notes


1. P. Tréguer *et al.*, *Science* **268**, 375 (1995).
2. S. W. Chisholm, *Nature* **407**, 685 (2000).
3. K. E. Apt, P. Behrns, *J. Phycol.* **35**, 215 (1999).
4. M. A. Borowitzka, *J. Biotech.* **70**, 313 (1999).
5. F. Chen, *Trends Biotechnol.* **14**, 421 (1996).
6. M. R. Droop, in *Algal Physiology and Biochemistry*, W. D. P. Stewart, Ed. (Univ. of California Press, Berkeley, 1974), pp. 530–559.
7. K. E. Apt, P. G. Kroth-Pancic, A. R. Grossman, *Mol. Gen. Genet.* **252**, 572 (1996).
8. L. A. Zaslavskaia, J. C. Lippmeier, P. G. Kroth, A. R. Grossman, K. E. Apt, *J. Phycol.* **36**, 379 (2000).
9. A. Falciatore, R. Cassotti, C. LeBlanc, C. Abrescia, C. Bowler, *Mar. Biotechnol.* **1**, 239 (1999).
10. K. E. Cooksey, *J. Phycol.* **10**, 253 (1974).
11. J. A. Hellebust, J. Lewin, in *The Biology of Diatoms*, D. Werner, Ed. (Univ. of California Press, Berkeley, 1977), pp. 169–197.
12. M. Mueckler, R. C. Hresko, M. Sato, *Biochem. Soc. Trans.* **25**, 951 (1997).
13. N. Sauer, W. Tanner, *FEBS Lett.* **259**, 43 (1989).
14. A. L. Kruckeberg, *Arch. Microbiol.* **166**, 283 (1996).
15. After 4 weeks in the dark, the transformants that grew were restreaked and maintained on 1.0% glucose. Liquid cultures were grown with 1.0% glucose at 20°C on an orbital shaker. All characterized transformants were generated from independent particle bombardments. Cells were grown at 20°C with continuous illumination at 75 μmol photons $m^{-2}$ $s^{-1}$ in Provasoli's enriched seawater medium with 10× the nitrogen and phosphorus by using Instant Ocean artificial seawater, at 0.5× concentration. Glucose was maintained between 5 and 10 g/liter. Growth rates were determined in 250-ml flasks (50 ml of media) with silicon foam closures. Daily samples measured cell numbers and nutrients. Flasks were stirred at 100 rpm. Fermentations were done in a 2-liter Applikont vessel by using an agitation rate of 100 rpm, dissolved oxygen was maintained at >20% saturation.
16. Cells in logarithmic phase growth were harvested, washed two times, and resuspended in fresh medium. Assays were initiated by adding unlabeled glucose and [D-$^{14}$C]glucose to 0.05 μCi/ml; the cells were maintained in the light. Samples were removed at 0, 2, 5, 10, and 15 min after the addition of labeled glucose. The cells were collected by filtration, washed with medium containing 1.0% unlabeled glucose, and transferred to scintillation vials.
17. L. A. Zaslavskaia *et al.*, unpublished data.
18. The cells were broken by using a MinibeadBeater by two cycles at full speed on ice. Cell membranes were pelleted by centrifugation at 100,000*g* for 30 min, solubilized in 2.0% SDS, resolved on 7.5% polyacrylamide gels, and transferred to nitrocellulose membranes.
19. T. Asano *et al.*, *J. Bio. Chem.* **266**, 24632 (1991).
20. L. L. Lu, A. Lundqvist, C. M. Zeng, C. Lagerquist, P. Lundahl, *J. Chromotagr.* **776**, 81 (1997).
21. Confocal microscopy was performed using a Nikon 60× N.a. = 1.2 water immersion objective on a Nikon TMD 200 inverted microscope outfitted with a BioRad MRC 1024 confocal head mounted in a Koehler configuration. EGFP was excited at 488 nm and visualized with a 522/25-nm bandpass filter. Plastid autofluorescence was excited at 456 nm and visualized with a 585-nm-long pass filter.
22. W. Chiu *et al.*, *Curr. Biol.* **6**, 325 (1996).
23. J. Haseloff, K. R. Siemering, D. C. Prasher, S. Hodge, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 2122 (1997).
24. A. Hallmann, M. Sumper, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 669 (1996).
25. H. Fischer, I. Robl, M. Sumper, N. Kroger, *J. Phycol.* **35**, 113 (1999).
26. P. G. Roessler, *J. Phycol.* **23**, 494 (1987).
27. J. A. Running, R. J. Huss, P. T. Olson, *J. Appl. Phycol.* **6**, 99 (1994).
28. R. J. Radmer, B. C. Parker, *J. Appl. Phycol.* **6**, 93 (1994).
29. J. A. Raven, P. G. Falkowski, *Plant Cell Environ.* **22**, 741 (1999).
30. M. R. Landry, *et al.*, *Marine Ecol. Progr. Ser.* **201**, 57 (2000).
31. E. Boyle, *Nature* **393**, 733 1998).
32. S. Takeda, *Nature* **393**, 777 (1998).


33. We thank D. Kyle and T. Allnutt for encouragement in the early parts of this work. Supported by NSF DMA-9560125 and funds from the Carnegie Institution of Washington.

20 February 2001; accepted 3 May 2001


# Telomere Position Effect in Human Cells


**Joseph A. Baur, Ying Zou, Jerry W. Shay,* Woodring E. Wright***



In yeast, telomere position effect (TPE) results in the reversible silencing of genes near telomeres. Here we demonstrate the presence of TPE in human cells. HeLa clones containing a luciferase reporter adjacent to a newly formed telomere express 10 times less luciferase than do control clones generated by random integration. Luciferase expression is restored by trichostatin A, a histone deacetylase inhibitor. Overexpression of a human telomerase reverse transcriptase complementary DNA results in telomere elongation and an additional 2- to 10-fold decrease in expression in telomeric clones but not control clones. The dependence of TPE on telomere length provides a mechanism for the modification of gene expression throughout the replicative life-span of human cells.


Most normal human cells lack the enzyme telomerase, which maintains telomeres, and as a consequence, telomeres shorten with each division until the cells reach replicative senescence (the Hayflick limit). This growth arrest is mediated by p53 and has been suggested to be the result of a DNA damage response to telomeres that have become too short (*1*–*3*). No mechanism has been demonstrated in vertebrates that can account for differences between young and old (but not yet senescent) cells. In *Saccharomyces cerevisiae*, telomere position effect (TPE) can result in the reversible silencing of a gene near a telomere by a mechanism that depends both on telomere length and on the distance to the gene (*4*–*6*). Because telomeres in most human cells shorten with age, TPE would provide a mechanism to incrementally alter phenotype with increasing cellular age (*7*). However, previous efforts to identify TPE in mammalian cells have not been successful (*8*–*10*). We demonstrate here the presence of TPE in human cells and that the strength of the silencing effect is dependent on telomere length.

We seeded de novo telomere formation in (telomerase-positive) HeLa cells by introducing a linear plasmid containing a luciferase reporter adjacent to 1.6 kb of telomere repeats (Web fig. 1) (*11*). Integration of a repeat-containing plasmid can result in breakage of the chromosome, followed by extension of the plasmid telomeric sequences by telomerase and loss of the distal chromosome fragment (*12*). Clones with a telomeric reporter were identified by Southern blotting of purified telomeres (Fig. 1A and Web fig. 2) (*11*) and confirmed by in situ hybridization (Fig. 1B). The mean length of the healed telomeres (after subtracting 3 kb of attached plasmid sequence) was estimated from Southern blots to be between 1.5 and 2 kb. Control clones were generated by transfection of an other-


Department of Cell Biology, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75390–9039, USA.

*To whom correspondence should be addressed. E-mail: Jerry.Shay@UTSouthwestern.edu (J.W.S) or Woodring.Wright@UTSouthwestern.edu (W.E.W).

wise identical linearized construct that lacked telomere repeats. As expected with plasmid transfections, there was a high degree of variation within each group. The clones with a telomeric reporter nonetheless expressed luciferase at a 10 times lower average level than did the clones with an internal integration site (Fig. 2A).

We next sought to demonstrate that the lower expression levels in telomeric clones were the result of heterochromatin formation rather than of damage to the transgene or the presence of a mixed population of clones. Heterochromatin in mammalian cells is normally dependent on histone deacetylation. We therefore investigated whether treatment with trichostatin A (TSA), a highly specific inhibitor of histone deacetylases (*13*), could eliminate the telomeric silencing effect we had observed. Telomeric and internal clones were treated with TSA. After treatment, both sets of clones expressed the reporter at an enhanced level, representing a 2.6 ± 0.4–fold increase for the internal clones and a 51 ± 37–fold increase for the telomeric clones (Fig. 2B). The initial difference in the level of luciferase expression is thus histone deacetylase–dependent. Enhancement of transgene expression by histone deacetylase inhibitors has been noted previously (*14*). Luciferase expression returned to pre-experiment levels within 72 hours after withdrawal of the TSA (*15*). Although the TSA dose used in these experiments is somewhat cytotoxic, the toxicity did not play a role in increasing luciferase expression, because nonspecific treatment with toxic doses of hygromycin led to a moderate decrease in luciferase activity (*15*).

We next extended telomeres in order to establish the length dependence of the observed silencing effect. Increasing the telomerase activity of HeLa cells by infection with a human telomerase reverse transcriptase (hTERT)–encoding retrovirus causes them to elongate their telomeres (Fig. 3A), as has been observed in several other cell lines (*16*). We observed an additional 2- to 10-fold decrease in luciferase activity after telomeric clones were infected with a telomerase-containing retrovirus, as compared to control, vector-only infections (Fig. 3B). This change was not observed in clones with an internal luciferase reporter. These results demonstrate that this effect shares some similarities with yeast TPE and provides a mechanism by which the expression of subtelomeric human genes could increase with replicative age.

The strongest evidence against the existence of mammalian TPE comes from a comparison of mRNA levels for a telomeric *neo* gene in subclones of SV40-transformed human fibroblasts with varying telomere lengths (*8*). This cell line uses the ALT (alternative lengthening of telomeres) pathway to maintain its telomeres, a phenotype that involves altered telomere biology and a substantial increase in total

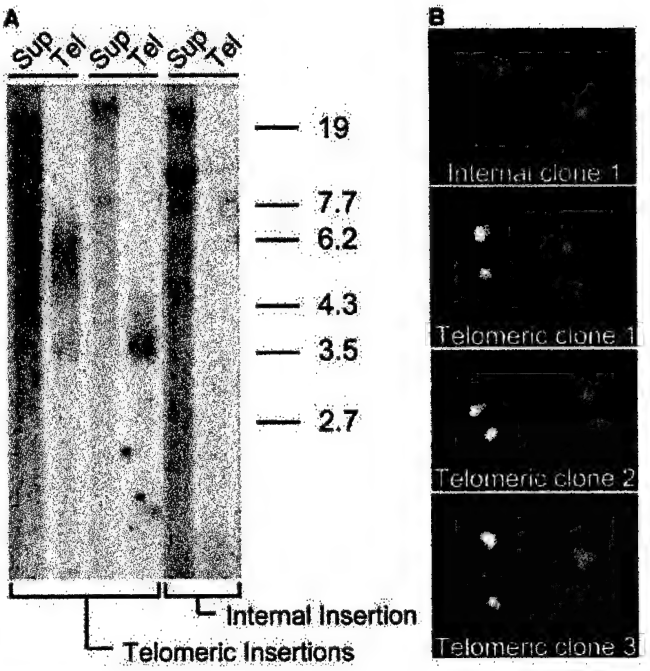



**Fig. 1.** Identification of telomeric clones by Southern blotting and in situ hybridization. **(A)** Genomic DNA was digested with Stu I, leaving the luciferase gene attached to the plasmid telomere sequences. Telomeres were then separated from bulk genomic DNA as described previously (*23*). Both the telomere fraction and the supernatant were separated on a 0.7% agarose gel, transferred to a Zeta-Probe blotting membrane (Bio-Rad, Hercules, California), and probed with luciferase sequences. Telomeric luciferase genes appear as a smear in the telomere fraction because of the heterogeneous lengths of the attached telomeres, whereas internally integrated genes appear as a discrete band in the supernatant fraction. Multiple integrations were noted in several of the internal control clones; however, the average was less than two (*11*). Markers shown are from λ DNA digested with *Sty* I (in kilobases). **(B)** Cells were fixed and probed simultaneously with the luciferase plasmid labeled with Spectrum Orange (Vysis, Downers Grove, Illinois), shown in red, and a fluorescein isothiocyanate–labeled oligonucleotide N3′-P5′ phosphoramidate probe complementary to telomere sequences [$(CCCTAA)_3$], shown in green. 4′,6′-diamidino-2-phenylindole staining is shown in blue. The top panel shows a clone with an internal integration site; the lower panels demonstrate the colocalization of the telomere and luciferase signals in three independent telomeric clones.

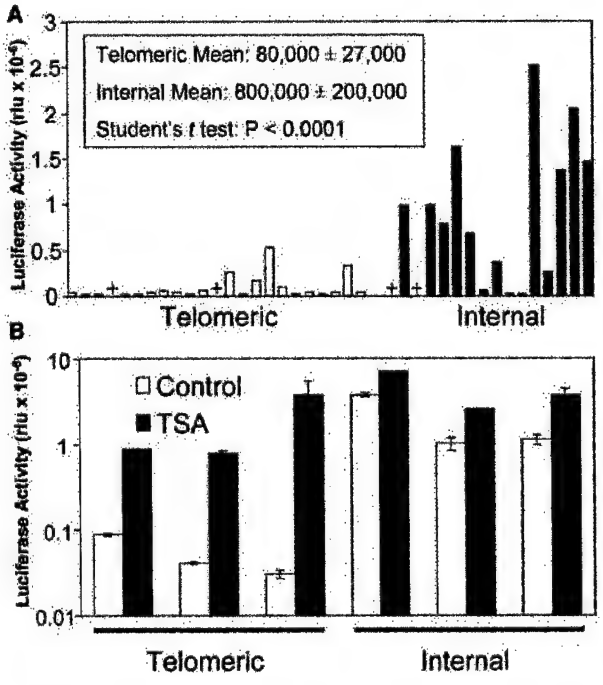



**Fig. 2.** Telomeric clones show a 10 times lower level of luciferase activity that is restored by a histone deacetylase inhibitor. **(A)** Puromycin-resistant clones were screened with a Luciferase Assay System (Promega, Madison, Wisconsin) on an Optocomp I luminometer (MGM Instruments, Hamden, Connecticut). The results for 23 telomeric and 15 internal integrations are shown. The plus signs indicate clones with a level of expression too low to be visible on this scale. **(B)** Silencing is relieved by the histone deacetylase inhibitor TSA. Three telomeric and three internal clones were treated with TSA (200 ng/ml) (Sigma, St. Louis, Missouri) for 24 hours. The medium was replaced, and the cells were incubated for an additional 24 hours before collection for luciferase assays. Note the switch to a logarithmic scale. rlu, relative light units.

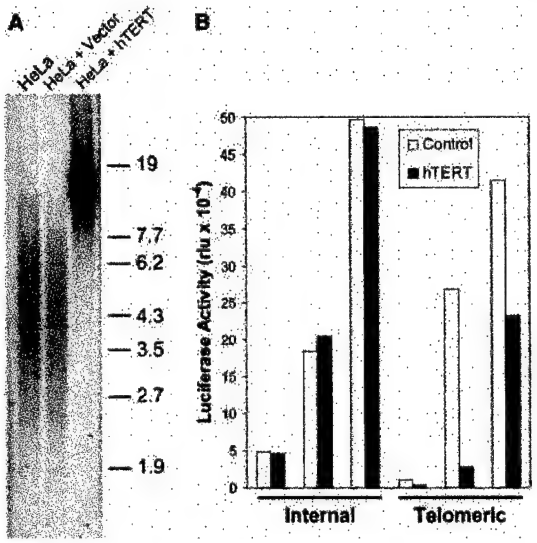



**Fig. 3.** Silencing in telomeric clones is enhanced by an increase in telomere length. (**A**) Infection of HeLa cells with an hTERT-encoding retrovirus causes telomere elongation, as demonstrated by terminal restriction fragment analysis. Mean telomere length increased from approximately 5 kb to almost 14 kb. Genomic DNA was digested with six restriction enzymes to degrade nonrepetitive sequences. Samples were then separated on a 0.7% agarose gel and probed with an oligonucleotide complementary to telomere repeats. Markers shown are λ *Sty* (in kilobases). (**B**) Telomeric clones infected with hTERT express 2 to 10 times lower levels of luciferase activity as compared to control, vector-only infections. Internal clones having comparable initial values retain full expression of the luciferase reporter after infection with hTERT.

telomeric DNA (*17*). It is possible that the extra telomeric sequences in ALT cells are titrating out factors essential for TPE, as has been observed in yeast (*18*), so that ALT cells might not exhibit TPE. Another report may have failed to identify TPE, because the healed telomere appears to have been extremely short and/or because it was located >50 kb from the nearest gene that could be examined (*9*). In at least one case, data consistent with a very mild mammalian TPE have been described (*19*), and the insertion of telomere repeats into an intron of the *APRT* gene of Chinese hamster cells was shown to cause a twofold reduction in the mRNA level (*20*).

A number of proteins have been reported to change in expression level as a function of the replicative age of the cell (*21*, *22*). The existence of TPE in mammalian cells raises the possibility that some presenescent changes could be "programmed" by the progressive shortening of telomeres with ongoing cell division, leading to altered patterns of gene expression that might affect both cell and organ function. It will be important to identify endogenous genes whose expression is influenced by telomere length in order to determine whether TPE actually influences the physiology of aging or cancer.

**References and Notes**

1. C. B. Harley, *Mutat. Res.* **256**, 271 (1991).
2. E. Hara, H. Tsurui, A. Shinozaki, S. Nakada, K. Oda, *Biochem. Biophys. Res. Commun.* **179**, 528 (1991).
3. J. W. Shay, O. M. Pereira-Smith, W. E. Wright, *Exp. Cell Res.* **196**, 33 (1991).
4. D. E. Gottschling, O. M. Aparicio, B. L. Billington, V. A. Zakian, *Cell* **63**, 751 (1990).
5. H. Renauld *et al.*, *Genes Dev.* **7**, 1133 (1993).
6. G. Kyrion, K. Liu, C. Liu, A. J. Lustig, *Genes Dev.* **7**, 1146 (1993).
7. W. E. Wright, J. W. Shay, *Trends Genet.* **8**, 193 (1992).
8. C. N. Sprung, L. Sabatier, J. P. Murnane, *Nucleic Acids Res.* **24**, 4336 (1996).
9. R. Ofir, A. C. Wong, H. E. McDermid, K. L. Skorecki, S. Selig, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 11434 (1999).
10. R. A. Bayne *et al.*, *Hum. Mol. Genet.* **3**, 539 (1994).
11. Supplementary Web material is available on *Science* Online at www.sciencemag.org/cgi/content/full/292/5524/2075/DC1
12. J. P. Hanish, J. L. Yanowitz, T. de Lange, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8861 (1994).
13. P. A. Marks, V. M. Richon, R. A. Rifkind, *J. Natl. Cancer Inst.* **92**, 1210 (2000).
14. T. Yamano *et al.*, *Mol. Ther.* **1**, 574 (2000).
15. J. A. Baur, J. W. Shay, W. E. Wright, unpublished data.
16. P. A. McChesney, D. L. Aisner, B. C. Frank, W. E. Wright, J. W. Shay, *Mol. Cell. Biol. Res. Commun.* **3**, 312 (2000).
17. T. M. Bryan, L. Marusic, S. Bacchetti, M. Namba, R. R. Reddel, *Hum. Mol. Genet.* **6**, 921 (1997).
18. E. A. Wiley, V. A. Zakian, *Genetics* **139**, 67 (1995).
19. H. Cooke, in *Telomeres*, E. Blackburn, Ed. (Cold Spring Harbor Laboratory Press, Plainview, NY, 1995), pp. 238–239.
20. A. E. Kilburn, M. J. Shea, R. G. Sargent, J. H. Wilson, *Mol. Cell. Biol.* **21**, 126 (2001).
21. M. Tresini, R. J. Pignolo, R. G. Allen, V. J. Cristofalo, *J. Cell Physiol.* **179**, 11 (1999).
22. L. Hayflick, in *Annual Review of Gerontology and Geriatrics*, C. Eisdorf, Ed. (Springer, New York, 1980), pp. 26–67.
23. W. E. Wright, V. M. Tesmer, K. E. Huffman, S. D. Levene, J. W. Shay, *Genes Dev.* **11**, 2801 (1997).
24. We thank C. Iucu for excellent technical support. Funding for this work was provided by the Ellison Medical Foundation (J.A.B. and J.W.S.), U.S. Department of Defense grant BC000422 (J.A.B. and J.W.S.), NIH grant AG07792 (W.E.W.), and the Geron Corporation, Menlo Park, CA.

7 March 2001; accepted 8 May 2001

# Requirement of *CHROMOMETHYLASE3* for Maintenance of CpXpG Methylation

**Anders M. Lindroth,[1]* Xiaofeng Cao,[1]* James P. Jackson,[1]* Daniel Zilberman,[1] Claire M. McCallum,[3] Steven Henikoff,[2,3] Steven E. Jacobsen[1]†**

Epigenetic silenced alleles of the *Arabidopsis SUPERMAN* locus (the *clark kent* alleles) are associated with dense hypermethylation at noncanonical cytosines (CpXpG and asymmetric sites, where X = A, T, C, or G). A genetic screen for suppressors of a hypermethylated *clark kent* mutant identified nine loss-of-function alleles of *CHROMOMETHYLASE3* (*CMT3*), a novel cytosine methyltransferase homolog. These *cmt3* mutants display a wild-type morphology but exhibit decreased CpXpG methylation of the *SUP* gene and of other sequences throughout the genome. They also show reactivated expression of endogenous retrotransposon sequences. These results show that a non-CpG DNA methyltransferase is responsible for maintaining epigenetic gene silencing.

Cytosine methylation plays a major role in determining the epigenetic expression state of eukaryotic genes. This methylation is most often found at the symmetrical dinucleotide CG (or CpG sites). CpG methylation is maintained by the well-studied DNMT1 subfamily of methyltransferases, which includes *Arabidopsis* MET1 (*1–3*). Methylation at sites other than CpG is also found in many organisms (*4*), but the mechanism by which this methylation is maintained is poorly understood. *Arabidopsis* can tolerate major disruptions in DNA methylation (*2, 3, 5*), making it useful for genetic analysis of methylation pattern-

[1]Department of Molecular, Cell and Developmental Biology, University of California, Los Angeles, CA 90095, USA. [2]Howard Hughes Medical Institute, [3]Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: jacobsen@ucla.edu

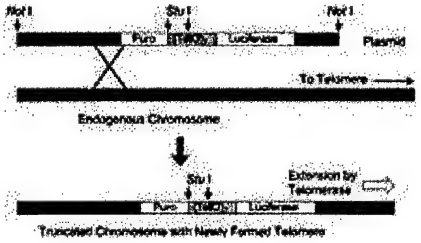
ABSTRACT
FULL TEXT

**Telomere Position Effect in Human Cells**

Joseph A. Baur, Ying Zou, Jerry W. Shay, and Woodring E. Wright

# Supplementary Material

**Supplemental Figure 1.** Generation of clones containing telomeric luciferase reporters. A plasmid containing a 1.6-kb tract of telomere repeats adjacent to a luciferase reporter was linearized using Not I and transfected into HeLa cells (*1-4*). Clones were recovered in which integration of the plasmid had caused the truncation of a chromosome and subsequent extension of the plasmid sequences to form a new telomere. $(TetO)_7$ represents seven tet operator sites fused to create a bidirectional tetracycline-responsive promoter. The indicated Stu I sites were used to analyze chromosomal insertion sites. The plasmid was designed to have a tet-inducible promoter driving both the puromycin selectable marker and a luciferase reporter. This would permit drug selection in the presence of a strong (induced) promoter, followed by growth and establishment of potential TPE under noninduced (weak promoter) conditions. The results shown in Web Fig. 2A were obtained after continuous growth under induced conditions. No significant change in the dose response or magnitude of the TPE was observed after selected telomeric and internal clones were cultured for 1 month in the presence of the tetracycline analog doxycycline (uninduced) and then tested for luciferase activity under graded levels of induction. We thus did not find any evidence for an influence of promoter strength on TPE in human cells within the range of our tet-off system, in contrast to yeast TPE which is abolished in the presence of a strong promoter (*5*, *6*). The remainder of the experiments were therefore carried out under doxycycline-free (fully induced) conditions.

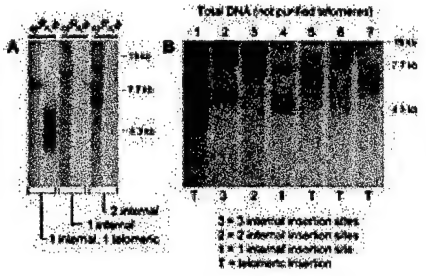



Medium version | Full size version

---

**Supplemental Figure 2.** Characterization of transgene integrations. (A) Additional clones were analyzed as described in Web Fig. 1. From left to right, they are a clone with both a telomeric and an internal integration, a clone with a single internal integration, and a clone with two internal integrations. Genomic DNA was digested with Stu I, leaving the luciferase gene attached to the plasmid telomere sequences (Web

Fig. 1). Telomeres were then separated from bulk genomic DNA by first annealing a biotinylated $(CCCTAA)_6$ oligonucleotide to the single-stranded overhang and then retrieving the telomeres using streptavidin beads as has been described previously (*7*). Both the telomere and supernatant (bulk genomic DNA) fractions were analyzed by Southern blotting. The fact that the signal seen in the telomere fraction for the first clone appears as a smear provides additional confirmation that the clone is telomeric, because the attached telomere sequences are heterogenous in length. (B) Multiple bands appear in some clones. Genomic DNA was digested using Stu I and analyzed by Southern blotting without prior separation of telomeres. Four telomeric clones with no internal sites (lanes 1, 5, 6, and 7), a single internal integration site (lane 4), two internal integration sites (lane 3), and three internal integration sites (lane 2) are shown.




Medium version | Full size version

---

**References and Notes**

1. The puromycin resistance gene was first inserted into the Pvu II site of the bidirectional tet-off vector pBI-2 by blunt cloning. The Bam H1-Bgl II fragment from pSXneo, containing 1.6 kb of telomeric repeats, proved unstable when cloned next to the luciferase gene of pBI-2 but was stable when placed into the Bgl II site next to the puromycin gene. The relative location of puromycin and luciferase was then flipped in order to obtain the final plasmid, pBX, in which the telomeric repeats were adjacent to luciferase. This was accomplished by ligation of the Nhe I-Hpa I fragment containing the luciferase/puromycin cassette from the intermediate plasmid to the Spe I-Bgl II fragment containing the rest of the plasmid backbone. A control vector for generating internal insertions was created by excising the $T_2AG_3$ repeats from pBX by Bst X1-Not I, blunting, and religating the plasmid. HeLa cells expressing the tetracycline-responsive transcriptional activator (tTA, tet-off) were used throughout these experiments. Transfections were carried out using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions.
2. U. Baron, S. Freundlieb, M. Gossen, H. Bujard, *Nucleic Acids Res.* **23**, 3605 (1995).
3. M. Gossen, H. Bujard, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5547 (1992).
4. Z. Zhong, L. Shiue, S. Kaplan, T. de Lange, *Mol. Cell. Biol.* **12**, 4834 (1992).
5. O. M. Aparicio, D. E. Gottschling, *Genes Dev* .**8**, 1133 (1994).
6. H. Renauld *et al.*, *Genes Dev.* 7, 1133 (1993).
7. W. E. Wright, V. M. Tesmer, K. E. Huffman, S. D. Levene, J. W. Shay, *Genes Dev.* **11**, 2801 (1997).

Chromosoma (2004) 112:240–246
DOI 10.1007/s00412-003-0269-x

RESEARCH ARTICLE

Joseph A. Baur · Jerry W. Shay · Woodring E. Wright

# Spontaneous reactivation of a silent telomeric transgene in a human cell line

Received: 26 September 2003 / Revised: 26 November 2003 / Accepted: 2 December 2003 / Published online: 20 January 2004


**Abstract** Subtelomeric reporter genes in human cells are silenced in a telomere length-dependent manner. Here we show that a subtelomeric reporter gene is expressed in only a subpopulation of cells within a clone and that this heterogeneity is generated by switching between expression states. We observed frequent reversion from the silenced state back to active expression. This process was more prominent for subtelomeric transgenes; however, we also observed cases of spontaneous reversion in control clones bearing the reporter at an internal site. We additionally show that treatment of these cells with 5-bromodeoxyuridine results in strong activation of the transgene. Although similar findings have been reported previously in mouse cells, this is, to our knowledge, the first direct observation of ongoing fluctuations in transcription in clonal populations of human cells. Our results suggest that this mechanism, as opposed to progressive silencing or a delayed fixing of expression states, accounts for the variegation in expression observed for subtelomeric transgenes in human cells. These data imply that telomere shortening during human aging could lead to stochastic activation of subtelomeric genes.

**Abbreviations** *BrdU:* 5-Bromodeoxyuridine · *CMV:* Cytomegalovirus · *DsRed2: Discosoma sp.* red fluorescent protein · *FACS:* Fluorescence activated cell sorting · *hTERT:* Human telomerase reverse transcriptase · *hTPE:* Human telomere position effect · *IRES:* Internal ribosome entry site · *TPE:* Telomere position effect · *TRF:* Terminal restriction fragment (analysis) · *TSA:* Trichostatin A

Communicated by V. Zakian

J. A. Baur · J. W. Shay · W. E. Wright (✉)
Department of Cell Biology,
University of Texas Southwestern Medical Center,
Dallas, TX 75390-9039, USA
e-mail: Woodring.Wright@UTSouthwestern.edu

## Introduction

Silencing near telomeres, termed telomere position effect (TPE), was first observed in *Drosophila* (Hazelrigg et al. 1984) and has since been studied extensively in *Saccharomyces cerevisiae* and other lower organisms (Gottschling et al. 1990; Tham and Zakian 2002). It is characterized by the semi-stable repression of subtelomeric reporter genes in a manner that is proportional to both the length of the telomere tract (Kyrion et al. 1993) and the proximity of the reporter to the telomere (Renauld et al. 1993). Telomere position effect was recently reported to affect an endogenous *S. cerevisiae* gene of unknown function (Vega-Palas et al. 2000). Although the involvement of telomeric silencing has been suggested in the regulation of surface antigen expression for several parasites (Horn and Cross 1995; Tham and Zakian 2002), a biologically relevant role for TPE has yet to be demonstrated.

One of the hallmarks of TPE in yeast is the spontaneous switching of a telomeric gene from an active to a silent state and vice versa. Our initial studies using a luciferase reporter to demonstrate silencing near human telomeres (human telomere position effect, or hTPE) (Baur et al. 2001) did not allow analysis at the single-cell level. We and others (Koering et al. 2002) have now confirmed the existence of human hTPE using fluorescent reporter genes in order to detect single cells. While the fraction of expressing cells varies widely in internal control clones, cell lines bearing a telomeric reporter consistently show expression in only a few percent of the cells. Such a pattern could conceivably be generated by several mechanisms, including slow progressive silencing, fixing of expression states at some point after the first few divisions (for example the chromatin structure at the integration site might take time to stabilize), or stochastic switching between expression states. While progressive silencing has been observed for transgenes in mammalian cells (Walters et al. 1996), available data on transgene expression at yeast telomeres (Gottschling et al. 1990) and at other variegating loci within mammalian cells

(Feng et al. 1999; Ronai et al. 1999) favor the third hypothesis. In the only published report examining TPE in human cells at the single-cell level, however, switching between expression states was not detected (Koering et al. 2002), raising the possibility that variegation in these cells is generated through one of the other two mechanisms. Here we show that in HeLa cells spontaneous reactivation of the transgene can be detected in initially negative subclones, demonstrating that heterogeneity is in fact generated through an ongoing stochastic process of switching between expression states.

## Materials and methods

### Generation of clones

The construction of luciferase-expressing clones has been described previously (Baur et al. 2001). To generate DsRed-expressing clones, the *AflIII*/*BfrI* fragment (containing the gene encoding the DsRed1 protein) from pDsRed1-N1 (Clontech, Palo Alto, Calif.) was blunt ligated into the *SmaI*/*HpaI* backbone from pSXneo1.6$T_2AG_3$ (Hanish et al. 1994) such that the Cytomegalovirus (CMV) promoter was placed at the base of the telomere repeats. Next, a blunted fragment containing an internal ribosome entry site (IRES) and the blasticidin resistance gene was ligated into the *HpaI* site in the same orientation as the DsRed1 protein. When DsRed2 became available, the new coding region was inserted by exchanging the DsRed fragment defined by *SalI*/*NotI* (this required a partial digest with *NotI*) and the resulting vector was designated pSXD2. The control vector lacking repeats was generated by blunt ligation after excision of the *ClaI*/*SacII* fragment. Vectors were linearized with *ClaI*/*PvuI* (with repeats) or *PvuI* alone (without repeats) and transfected into HeLa cells using FuGENE 6 transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. Stable clones were obtained by ring-cloning after selection in 1 μg/ml blasticidin for 1 week. Individual clones were isolated by placing a glass ring over them (sealed with vacuum grease) and transferred to separate dishes by standard trypsinization methods.

### Analysis of clones

Cells were observed primarily on a Zeiss Axiovert 100M inverted microscope attached to a MacIntosh G4 computer using Openlab imaging software. Scanning was performed on a FACScan (Becton Dickinson, San Jose, Calif.).

#### *Initial subcloning*

Cells were plated on several 10 cm dishes at a density of 300 cells/dish. The next day, well-isolated subclones were selected and photographed. Bright-field and fluorescent images were collected at regular intervals in order to follow changes in the expression pattern as each clone grew.

#### *Subcloning into 96-well plates*

Single cells were sorted into each well of a 96-well plate using a MoFlo high-speed cell sorter (Cytomation, Fort Collins, Colo.) and a FACStar Plus (Becton Dickinson, San Jose, Calif.). Sorting into 96-well plates was an automated feature of the cell sorters. Medium (100 μl) was placed in each well prior to sorting and cells were selected by size only (to avoid fragments and doublets). Wells containing cells were identified by microscopy the following day (2–4 cell stage) and the remainder of each well was checked carefully to ensure that no other cells were present. Cells were observed every 2–3 days thereafter for at least 3 weeks.

### Retroviral infections

The amphotrophic retroviral packaging cell line PA317 (Miller 1990) was infected using supernatants from PE501 cells that had been transiently transfected with retroviral plasmid DNA. Following selection, supernatants were harvested from PA317 cells, purified by passage through a 0.45 μm sterile filter, and stored at –80°C for later use. Infection of target cells was carried out by 8–16 h exposure to supernatant diluted 1:2 in regular medium with 4 μg/ml (final) polybrene (Sigma, St. Louis, Mo.). Cells were then allowed to recover for 12–24 h before selection.

### Terminal restriction fragment (TRF) analysis

Cells were suspended in 100 mM NaCl, 100 mM EDTA, and 10 mM TRIS, pH 8 at 20,000 cells/μl. Genomic DNA was extracted by bringing the final concentrations of Triton X-100 and proteinase K up to 1% and 2 mg/ml, respectively, and incubating for 12 h at 55°C, followed by inactivation of proteinase K at 70°C for 30 min. Samples were then dialyzed overnight against TE, pH 8. After dialysis, 1 μg DNA was digested with a mixture of six restriction enzymes (*AluI*, *CfoI*, *HaeI*, *HinfI*, *MspI*, and *RsaI*) with 4 bp target sites and run on a 0.7% agarose gel overnight at 70 V. The gel was denatured for 20 min in 0.5 M NaOH and 1.5 M NaCl, rinsed 10 min in water, dried 1 h at 55°C, neutralized for 15 min in 1.5 M NaCl and 0.5 M TRIS, pH 8, and probed with $^{32}$P-labeled $(T_2AG_3)_4$. After washing in 2×SSC for 15 min and 0.1×SSC with 0.1% SDS twice for 10 min, the gel was exposed to a Phosphor screen and analyzed using a Storm 860 PhosphorImager (Molecular Dynamics/Amersham, Piscataway, N.J.).

### 5-Bromodeoxyuridine treatment

Cells were treated with 50 μM 5-bromodeoxyuridine (BrdU) for 2–5 days in regular medium. A small decrease in growth rate was noted; however, toxicity was dramatically reduced as compared with trichostatin A (TSA) treatment.

## Results

In order to detect switching in human cells, we used a linearized plasmid containing telomere repeats (Hanish et al. 1994) to construct a series of clones bearing the fluorescent reporter DsRed2 either next to a newly formed telomere, or randomly integrated into the genome (Fig. 1A). Clones were verified by Southern blot hybridization and those containing multiple copies of the DsRed2 construct were discarded. While the number of cells expressing the reporter varied from 0 to nearly 100% in our internal controls (random integration), all of the telomeric clones recovered in our initial experiment expressed the DsRed2 protein in only a few percent of the cells (Fig. 1C), consistent with the findings of Koering et al. (2002). The fact that expression near human telomeres can be increased by the histone deacetylase inhibitor TSA (Baur et al. 2001; Koering et al. 2002), but not by the demethylating agent 5-azacytidine (Koering et al. 2002), suggests that a methylation-independent process causes the majority of the silencing in these cells.

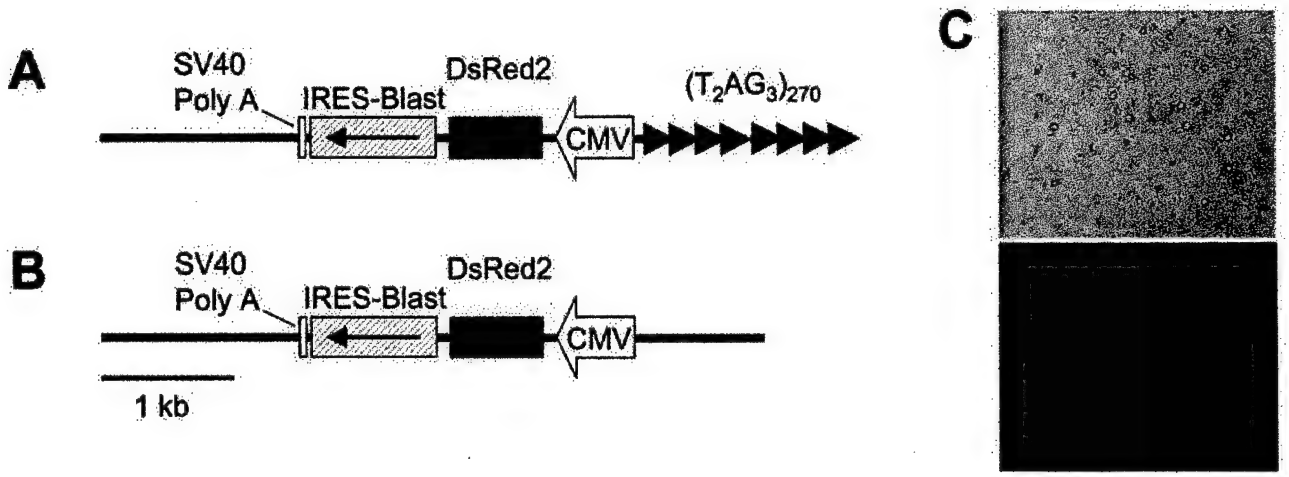



**Fig. 1 A, B** Structure of the constructs used in the generation of clones. Both vectors were transfected into HeLa cells in linear form using FuGENE 6 transfection reagent (Roche, Basel, Switzerland). In the chromosome truncation vector (**A**), the CMV promoter was located approximately 80 nucleotides from the base of the $T_2AG_3$ repeats. The fluorescent protein DsRed2 was transcribed away from the telomere and an internal ribosome entry site (*IRES*) was used to allow expression of the blasticidin resistance gene (*Blast*) from the same transcript. Transcription was terminated using the SV40 polyadenylation signal (*SV40 Poly A*). In the vector used to generate internal (control) integrations (**B**), the $T_2AG_3$ repeats were replaced with an additional 1 kb of plasmid sequences. **C** Bright field and fluorescent images of a representative clone bearing a telomeric DsRed2 reporter

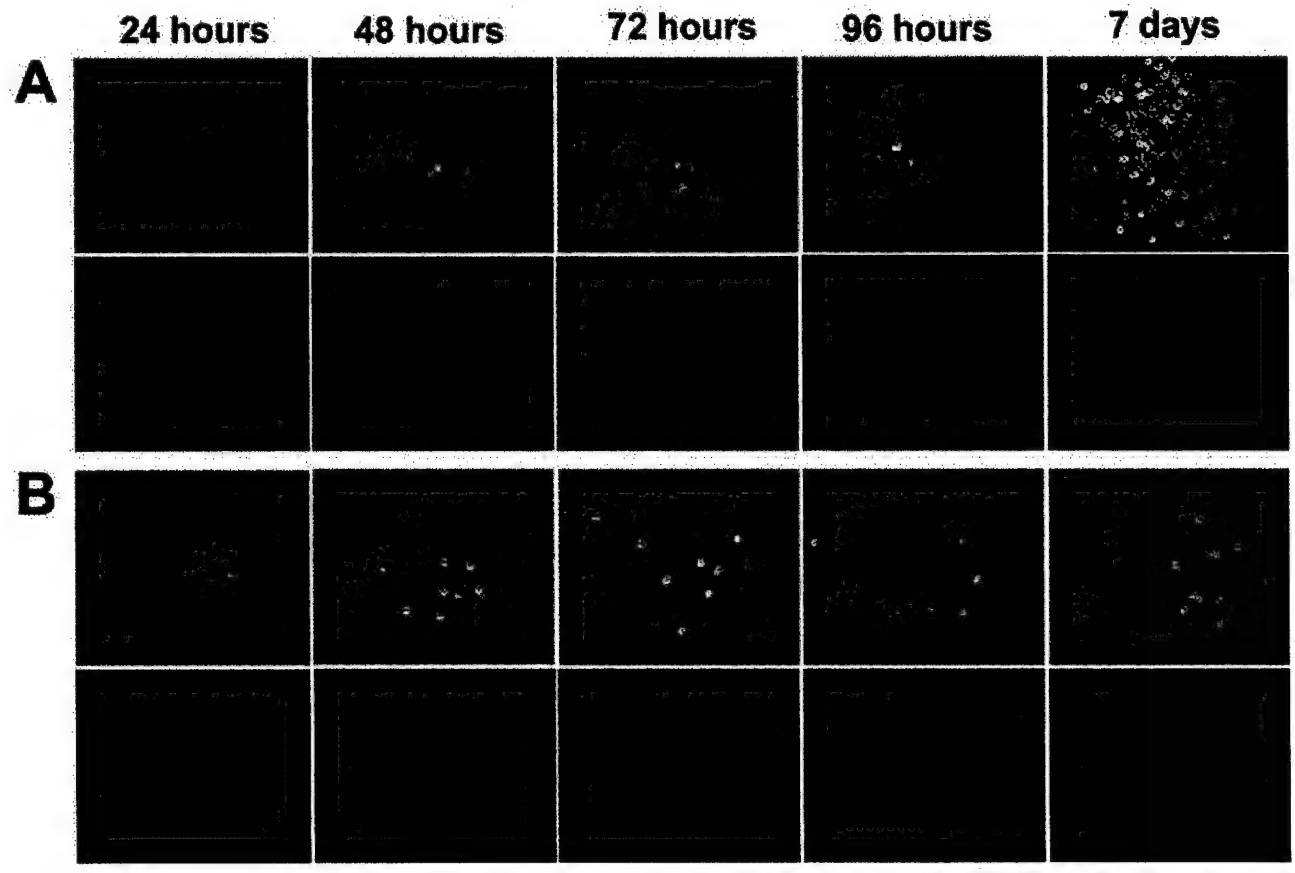



**Fig. 2A, B** Development of heterogeneity within subclones bearing a telomeric reporter. Paired bright field and fluorescent images are shown for two different subclones at five time points. While all cells initially express a uniform level of the DsRed2 protein (24 and 48 h time points), significant differences become apparent by 72 h (**B**) or 96 h (**A**), and both show obvious heterogeneity by 1 week

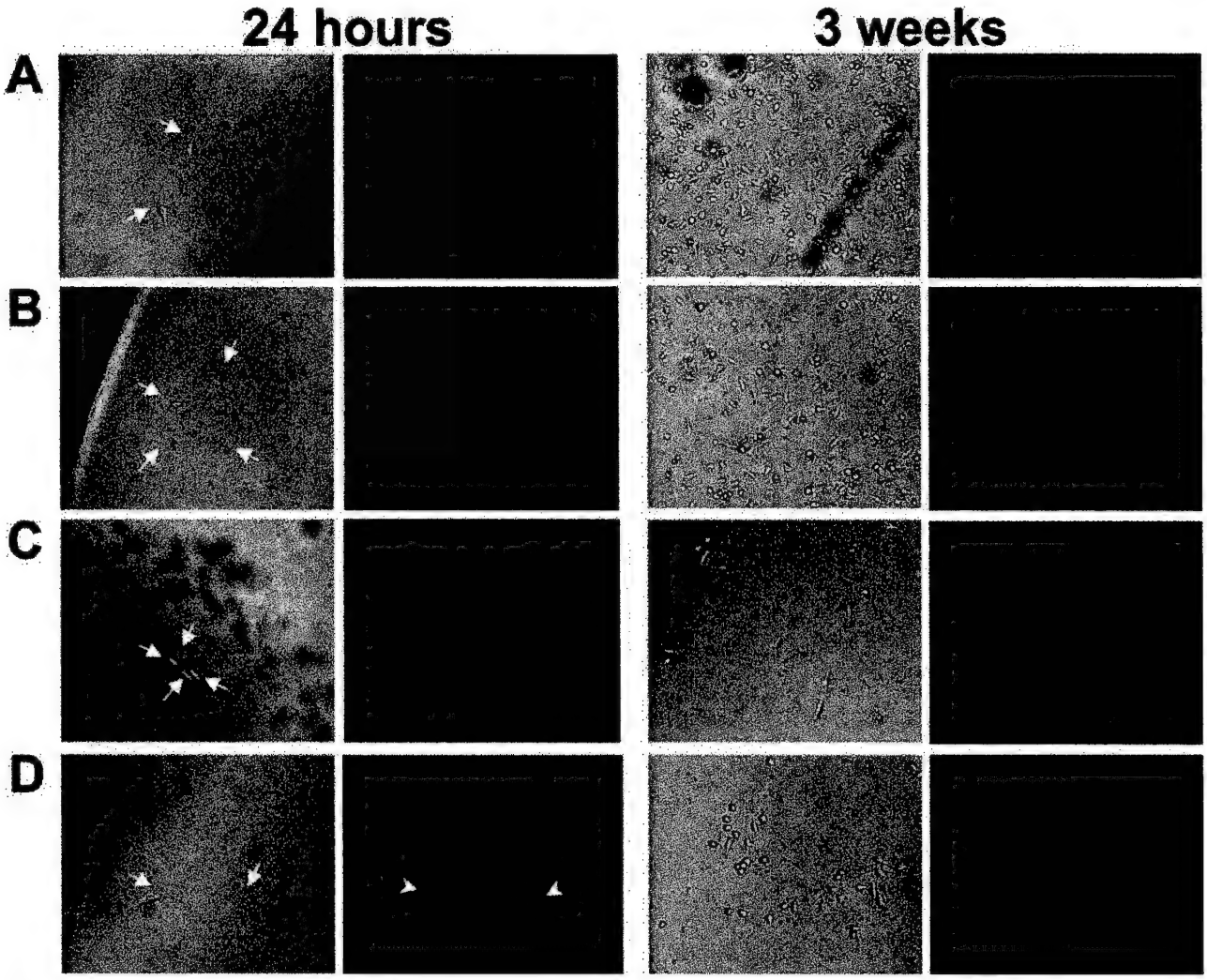



**Fig. 3A–D** Spontaneous reversal of silencing in cells bearing a telomeric reporter gene. Each row represents a different subclone derived from a single parental clone in which the gene for DsRed2 fluorescent protein had been placed next to a newly formed telomere. Subclones were initially negative (**A–C**) or very weak (**D**) for DsRed2 expression but by 3 weeks after subcloning most had developed sporadic expression in a small fraction of cells, resembling the pattern of expression in the parental cell line. *Arrows* indicate the positions of cells in the original bright field images. *Arrowheads* indicate weakly positive cells in the fluorescent image of subclone **D**

A previous attempt to demonstrate switching in human cells using fluorescent activated cell (FAC) sorted populations was not successful (Koering et al. 2002). Because FAC sorting cannot produce a 100% pure population of positive or negative cells, we chose to examine the progeny of single positive or negative cells by florescence microscopy. The presence of cells of opposite phenotype in a growing subclone would clearly indicate that a switching event had occurred. We subcloned cells bearing a single telomeric insertion of the DsRed2 construct and followed their growth and expression of the transgene (Fig. 2). In each case, cells that were initially positive gave rise to mixed populations within one week. Positive cells arising from initially negative cells were observed only rarely during this first experiment and the possibility that these rare cells were the result of contamination by floating cells could not be ruled out.

To demonstrate more clearly spontaneous reactivation of the transgene, we subcloned the progeny of a single cell into 96-well plates to eliminate the possibility of cross-contamination by floating cells. Growth of each subclone was then followed by fluorescence microscopy for an extended period of time. Each subclone was first observed at the 1- to 4-cell stage and was grown for approximately 20 population doublings. In 18 of 19 subclones that were initially negative, DsRed2-expressing cells were detected within 1 to 2 weeks (Fig. 3A–C), with 11 of the subclones becoming similar in appearance to the parent clone by week 3. Subclones that were initially weakly positive gave both strongly positive and completely negative cells within 1 to 2 weeks (Fig. 3D). Our

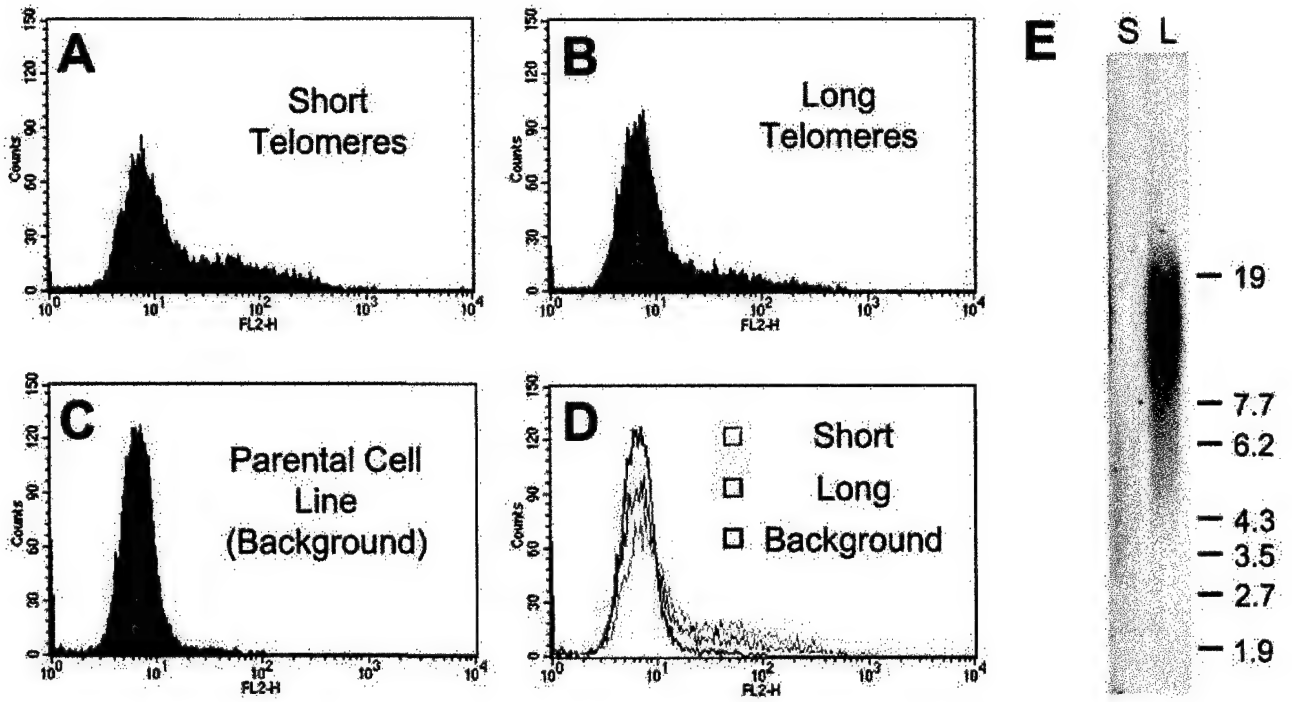



**Fig. 4A–D** Elongation of telomeres by hTERT overexpression decreases expression of DsRed2 in telomeric clones. Clones were infected with an empty vector (pBabe) or a retrovirus encoding the telomerase catalytic component (hTERT). Fluorescence-activated cell sorting (FACS) analysis is shown for a cell line bearing a telomeric DsRed2 reporter before telomere elongation (**A**) or after telomere elongation (**B**). FACS analysis of the parental cell line, which does not express DsRed2, is shown in **C**, and the three histograms are overlaid in **D**. **E** Terminal restriction fragment (TRF) analysis of the clones with long (*L*) and short (*S*) telomeres. Genomic DNA was digested with a mixture of six restriction enzymes, separated on a 0.7% agarose gel, and probed with an oligonucleotide complementary to telomere repeats. Markers shown are $\lambda$ *Sty* fragments (in kilobases)

data suggest that TPE in these cells resembles the variegation observed at yeast telomeres (Gottschling et al. 1990) and in the cells of transgenic mice (Rakyan et al. 2002), where all-or-none patterns of switching predominate over gradual changes in gene expression (Robertson et al. 1995; Ronai et al. 1999). The fact that Koering et al. (2002) did not observe fluctuations in expression at human telomeres likely indicates that cell type, the site of chromosome truncation, and/or the direction of transcription can influence the rate of switching.

We previously demonstrated, using a luciferase reporter, that telomere elongation enhances the silencing of telomeric transgenes (Baur et al. 2001). Elongation of telomeres in cells bearing a telomeric DsRed2 reporter also leads to a reduction in transgene expression (Fig. 4). The reduction in reporter expression is manifested primarily as a decrease in the fraction of positive cells, as opposed to intensity per cell, consistent with previous reports in human cells (Walters et al. 1995, 1996). This suggests that telomere elongation enhances silencing through effects on the heritability of the two states, as opposed to reducing the level of expression in the active state.

5-Bromodeoxyuridine has been shown to suppress position-effect variegation at internal loci in HeLa cells by a mechanism that remains poorly understood (Suzuki et al. 2001). Treating clones bearing a telomeric reporter gene with BrdU resulted in a loss of variegation and an enhancement of transgene expression. Relief of silencing for a telomeric DsRed2 reporter was observed after a 72 h incubation in medium containing 50 $\mu$M BrdU (Fig. 5A). Silencing was also relieved in low-expressing internal controls and in cells bearing a luciferase reporter at the telomere (Fig. 5B). It has previously been reported that silencing near telomeres can be relieved using the histone deacetylase inhibitor TSA (Baur et al. 2001; Koering et al. 2002); however, BrdU may be a preferable alternative in the future since it is significantly less toxic at the effective dose (data not shown). The fact that BrdU suppresses position-effect variegation of transgenes at both internal and telomeric loci suggests a common mechanism of repression.

As early as 1972, Lin and Riggs were able to show that incorporation of BrdU into DNA increases the binding affinities of certain proteins, including histone H1 (Lin and Riggs 1972; Lin et al. 1976). Since histone H1 binds to AT-rich regions of DNA that mediate scaffold/matrix attachment and are known to have dramatic effects on neighboring promoters (Blasquez et al. 1989; Stief et al. 1989; Klehr et al. 1991), it was hypothesized that BrdU might affect binding of DNA to the nuclear matrix (Suzuki et al. 2001). Indeed, BrdU has recently been

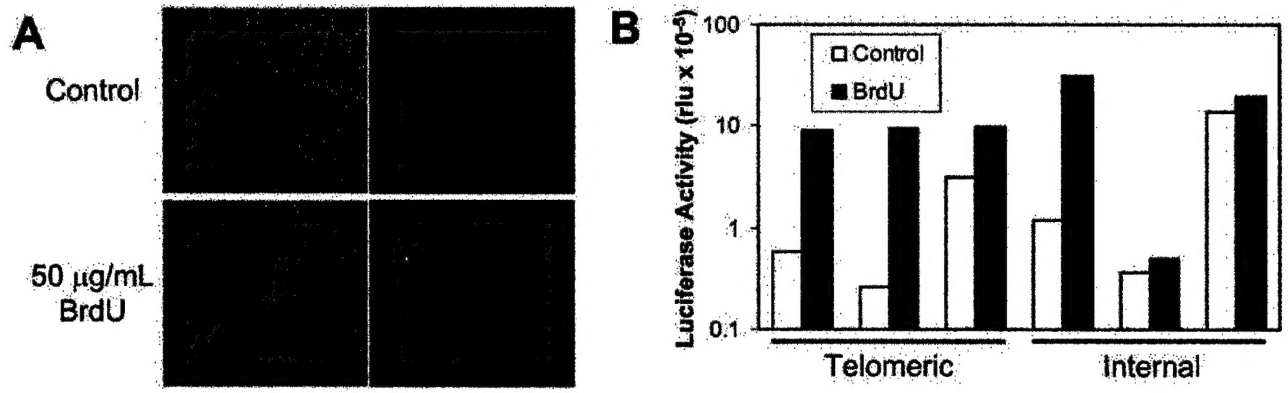



**Fig. 5** Loss of telomeric silencing in the presence of 5-bromodeoxyuridine (*BrdU*). Cells bearing a telomeric reporter were grown in regular medium (*Control*) or medium supplemented with 50 μg/ml BrdU for 72 h before analysis. **A** Expression of DsRed2 was dramatically upregulated in the presence of BrdU and toxicity was minimal compared with that observed when the cells were treated with trichostatin A (TSA). As with TSA (Baur et al. 2001), BrdU also affected silencing in low-expressing internal control clones (data not shown). **B** Silencing of a luciferase reporter was relieved after BrdU treatment in both telomeric and internal clones. Representative data are presented showing that expression after treatment is higher and more uniform between clones. Occasional resistant clones were noted among the internal controls (*middle*) and may represent cases in which methylation or rearrangement of the gene has occurred

shown to enhance binding of scaffold/matrix attachment regions to the nuclear matrix, and, moreover, the effects of BrdU are potentiated by competitive inhibitors of histone H1 binding (Suzuki et al. 2001; Ogino et al. 2002) . These findings are especially intriguing in the light of the fact that human telomeres are attached to the nuclear matrix via their TTAGGG repeats (de Lange 1992). Elucidation of the role played by the nuclear matrix in hTPE will be an interesting area for future studies.

## Discussion

We have shown in human cells that a clonal population derived from a cell bearing a telomeric reporter gene can acquire a variegated phenotype through a stochastic process of switching between expression states. Taken together with previous studies of transgene expression in mammalian cells (Walters et al. 1995, 1996; Dorer 1997; Feng et al. 1999; Ronai et al. 1999), our data suggest that many repressive loci within the human genome may be similar in this respect. The fact that expression at both telomeric and internal loci is enhanced by treatment with either TSA or BrdU is also suggestive of a common silencing mechanism.

At present, TPE is distinguished from other repressive effects in human cells only by its dependence on telomere length and its relative strength (tenfold lower expression on average relative to internal loci) (Baur et al. 2001). We observed 100% penetrance of variegation within the first 2 weeks of clonal growth in telomeric clones as opposed to ~50% in internal controls (our unpublished results), although at later time points many of the internal controls that were initially uniform in expression did become more heterogeneous. Some suggestive evidence has been presented that heterochromatin protein 1 (HP1) may be specifically involved in telomeric silencing (Koering et al. 2002). Overexpression of HP1 was previously shown to enhance variegation at centromeric loci while suppressing variegation at non-centromeric loci (Festenstein et al. 1999). This suggests that variegation may occur by at least two distinct mechanisms in mammalian cells despite the similar outward appearances of various mammalian position effects. It will be interesting in the future to see whether or not a specific set of proteins is involved in telomeric silencing.

A change in the expression of endogenous human genes regulated by telomere length has not yet been demonstrated. The present results suggest that if such regulation occurs, it will likely be manifested primarily by an "all-or-none" change in expression in a small fraction of cells rather than a quantitative increase in expression per cell. However, detailed studies in yeast have revealed regulatory elements at endogenous telomeres that produce marked differences in transgene behavior as compared with truncated chromosome ends (Fourel et al. 1999; Pryde and Louis 1999). Whether or not endogenous human telomeres possess elements that might alter or block TPE remains to be seen.

It has been suggested that the high frequency of duplications and rearrangements in subtelomeric regions may facilitate "rapid adaptive evolution" (Mefford and Trask 2002). This hypothesis is supported by the finding that a block of subtelomeric sequence containing three olfactory receptor genes is duplicated polymorphically on at least 14 different chromosome ends in humans but is single copy in non-human primates (Trask et al. 1998). Telomere position effect may contribute to this process through silencing of subtelomeric genes and the production of a variegated phenotype, allowing the majority of cells to progress through potentially harmful genetic intermediates while continuously sampling the products

of rearranged genes. A similar role has been proposed for prion proteins in the generation of genetic diversity (True and Lindquist 2000). Collapse of the silent domains near human telomeres (i.e. loss of silencing due to telomere shortening) might be predicted to trigger inappropriate expression of a host of rearranged subtelomeric genes as part of the aging process. It is also possible that the telomere shortening accompanying cell division might provide a method for timing changes that occur over many years in long-lived organisms. It is difficult to imagine how conventional timing mechanisms (biochemical oscillations, lunar cycles of changes, changing gradients during early development) could be adapted to count decades. Telomere position effect-regulated changes in gene expression produced by slow but ongoing cell turnover could provide such a mechanism. Clearly further study is required to elucidate the role (if any) of TPE in human gene regulation, aging, and disease.

**Acknowledgements** This work was supported by Department of Defense grant BC000422 (J.A.B.) and National Institutes of Health grant AG07792 (W.E.W. and J.W.S.)

## References

Baur JA, Zou Y, Shay JW, Wright WE (2001) Telomere position effect in human cells. Science 292:2075–2077

Blasquez VC, Xu M, Moses SC, Garrard WT (1989) Immunoglobulin kappa gene expression after stable integration. I. Role of the intronic MAR and enhancer in plasmacytoma cells. J Biol Chem 264:21183–21189

de Lange T (1992) Human telomeres are attached to the nuclear matrix. EMBO J 11:717–724

Dorer DR (1997) Do transgene arrays form heterochromatin in vertebrates? Transgenic Res 6:3–10

Feng YQ, Alami R, Bouhassira EE (1999) Enhancer-dependent transcriptional oscillations in mouse erythroleukemia cells. Mol Cell Biol 19:4907–4917

Festenstein R, Sharghi-Namini S, Fox M, Roderick K, Tolaini M, Norton T, Saveliev A, Kioussis D, Singh P (1999) Heterochromatin protein 1 modifies mammalian PEV in a dose- and chromosomal-context-dependent manner. Nat Genet 23:457–461

Fourel G, Revardel E, Koering CE, Gilson E (1999) Cohabitation of insulators and silencing elements in yeast subtelomeric regions. EMBO J 18:2522–2537

Gottschling DE, Aparicio OM, Billington BL, Zakian VA (1990) Position effect at *S. cerevisiae* telomeres: reversible repression of Pol II transcription. Cell 63:751–762

Hanish JP, Yanowitz JL, de Lange T (1994) Stringent sequence requirements for the formation of human telomeres. Proc Natl Acad Sci U S A 91:8861–8865

Hazelrigg T, Levis R, Rubin GM (1984) Transformation of white locus DNA in drosophila: dosage compensation, zeste interaction, and position effects. Cell 36:469–481

Horn D, Cross GA (1995) A developmentally regulated position effect at a telomeric locus in *Trypanosoma brucei*. Cell 83:555–561

Klehr D, Maass K, Bode J (1991) Scaffold-attached regions from the human interferon beta domain can be used to enhance the stable expression of genes under the control of various promoters. Biochemistry 30:1264–1270

Koering CE, Pollice A, Zibella MP, Bauwens S, Puisieux A, Brunori M, Brun C, Martins L, Sabatier L, Pulitzer JF, Gilson E (2002) Human telomeric position effect is determined by chromosomal context and telomeric chromatin integrity. EMBO Rep 3:1055–1061

Kyrion G, Liu K, Liu C, Lustig AJ (1993) RAP1 and telomere structure regulate telomere position effects in *Saccharomyces cerevisiae*. Genes Dev 7:1146–1159

Lin SY, Riggs AD (1972) Lac operator analogues: bromodeoxyuridine substitution in the lac operator affects the rate of dissociation of the lac repressor. Proc Natl Acad Sci U S A 69:2574–2576

Lin S, Lin D, Riggs AD (1976) Histones bind more tightly to bromodeoxyuridine-substituted DNA than to normal DNA. Nucleic Acids Res 3:2183–2191

Mefford HC, Trask BJ (2002) The complex structure and dynamic evolution of human subtelomeres. Nat Rev Genet 3:91–102

Miller AD (1990) Retrovirus packaging cells. Hum Gene Ther 1:5–14

Ogino H, Fujii M, Satou W, Suzuki T, Michishita E, Ayusawa D (2002) Binding of 5-bromouracil-containing S/MAR DNA to the nuclear matrix. DNA Res 9:25–29

Pryde FE, Louis EJ (1999) Limitations of silencing at native yeast telomeres. EMBO J 18:2538–2550

Rakyan VK, Blewitt ME, Druker R, Preis JI, Whitelaw E (2002) Metastable epialleles in mammals. Trends Genet 18:348–351

Renauld H, Aparicio OM, Zierath PD, Billington BL, Chhablani SK, Gottschling DE (1993) Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by SIR3 dosage. Genes Dev 7:1133–1145

Robertson G, Garrick D, Wu W, Kearns M, Martin D, Whitelaw E (1995) Position-dependent variegation of globin transgene expression in mice. Proc Natl Acad Sci U S A 92:5371–5375

Ronai D, Berru M, Shulman MJ (1999) Variegated expression of the endogenous immunoglobulin heavy-chain gene in the absence of the intronic locus control region. Mol Cell Biol 19:7031–7040

Stief A, Winter DM, Stratling WH, Sippel AE (1989) A nuclear DNA attachment element mediates elevated and position-independent gene activity. Nature 341:343–345

Suzuki T, Yaginuma M, Oishi T, Michishita E, Ogino H, Fujii M, Ayusawa D (2001) 5-Bromodeoxyuridine suppresses position effect variegation of transgenes in HeLa cells. Exp Cell Res 266:53–63

Tham WH, Zakian VA (2002) Transcriptional silencing at Saccharomyces telomeres: implications for other organisms. Oncogene 21:512–521

Trask BJ, Friedman C, Martin-Gallardo A, Rowen L, Akinbami C, Blankenship J, Collins C, Giorgi D, Iadonato S, Johnson F, Kuo WL, Massa H, Morrish T, Naylor S, Nguyen OT, Rouquier S, Smith T, Wong DJ, Youngblom J, van den Engh G (1998) Members of the olfactory receptor gene family are contained in large blocks of DNA duplicated polymorphically near the ends of human chromosomes. Hum Mol Genet 7:13–26

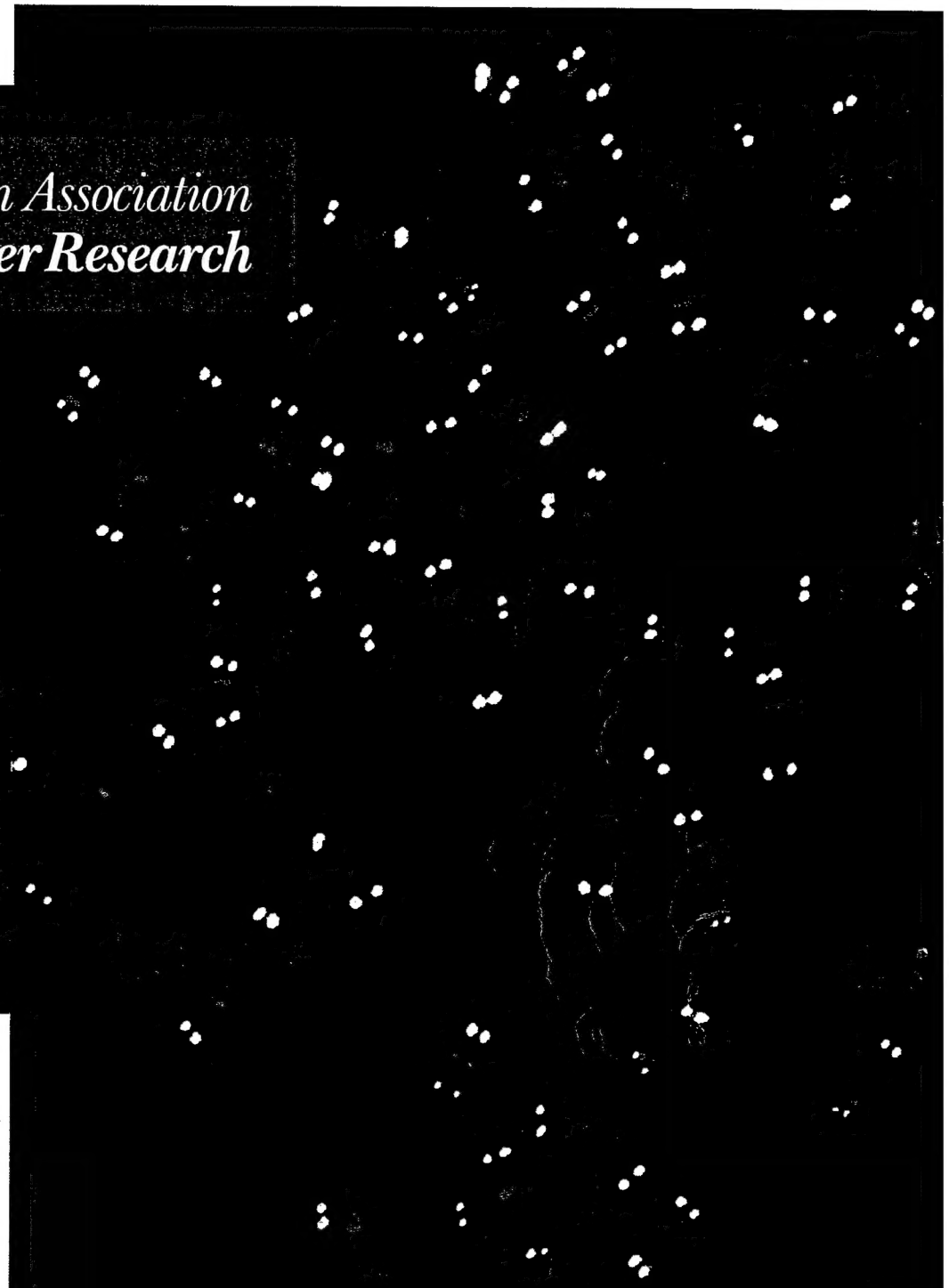
True HL, Lindquist SL (2000) A yeast prion provides a mechanism for genetic variation and phenotypic diversity. Nature 407:477–483

Vega-Palas MA, Martin-Figueroa E, Florencio FJ (2000) Telomeric silencing of a natural subtelomeric gene. Mol Gen Genet 263:287–291

Walters MC, Fiering S, Eidemiller J, Magis W, Groudine M, Martin DI (1995) Enhancers increase the probability but not the level of gene expression. Proc Natl Acad Sci U S A 92:7125–7129

Walters MC, Magis W, Fiering S, Eidemiller J, Scalzo D, Groudine M, Martin DI (1996) Transcriptional enhancers act in cis to suppress position-effect variegation. Genes Dev 10:185–195

AACR American Association for Cancer Research



*An AACR Special Conference in Cancer Research*

***Conference Chairpersons***

**Jerry W. Shay**
UT Southwestern Medical Center
Dallas, TX
**Elizabeth H. Blackburn**
University of California
San Francisco, CA

# The Role of Telomeres and Telomerase in Cancer

**December 7–11, 2002**
**Hyatt Regency San Francisco**
**San Francisco, California**

***Conference Proceedings***

**Non-Canonical Telomeric Sequences in the Regulation of Replicative Aging**

Woodring E. Wright and Ying Zou
Department of Cell Biology
University of Texas Southwestern Medical Center, Dallas, TX 75390

A central unexplained aspect of the two-stage (M1/M2) model of replicative aging is the mechanism by which cells deal with the shortest telomeres during their extended lifespan. If telomeres are sufficiently short to induce a DNA damage response at M1, why are apoptotic responses delayed for many doublings until M2? We initially considered two possibilities. In the presence of viral oncoproteins, a cell might tolerate a few chromosome fusions and become aneuploid. In this situation, M2 would only occur when the number of chromosomes with unprotected ends became sufficiently great to induce apoptosis. A second possibility is that gene conversion events might permit short telomeres to invade longer telomeres and become extended. M2 would occur when the longest telomeres became too short to be effective donors. Both of these hypotheses predict that the number of doublings between M1 and M2 should be reduced in cells with a more homogeneous telomere length (which can be induced by the transient expression of telomerase that preferentially elongates the shortest telomeres). Contrary to expectations, the same approximately 15 doubling extension of lifespan was observed in BJ-E6/E7 cells and BJ-E6/E7 cells following the transient expression of telomerase.
Karyotype and telomeric Q-fish analysis was performed on the HPV E6/E7cells in order to determine how the cells handled "too short telomeres" during the period of extended lifespan. The population doubling level at which M1 would have occurred in the absence of E6/E7 coincided with the appearance of telomeric end-associations. 90% of the end-associations involved the ten shortest telomeres, with the frequency of abnormalities being uniformly distributed among these ten. This implies that rather than having a single limiting chromosome, BJ cells use roughly 10% of their ends to monitor telomere length. The fact that short ends preferentially formed end-associations with other short ends either implies that a cell can tolerate an unprotected end until a second end with which it can associate becomes unprotected, or that the protection of short ends is metastable, so that a too-short end needing a fusion partner would preferentially destabilize another short end. The appearance of telomere associations did not produce significant aneuploidy in these E6/E7 expressing cells, and cells exhibited no change in their growth rates even when 40%-80% of the metaphases exhibited a telomere association. Dicentrics (end-associations with a detectable structural abnormality) appeared approximately 5-7 doublings after end-associations and their frequency correlated strongly with progressively increasing population doubling times leading to crisis.
These results suggest that there may be a qualitative difference in the state of short telomeres that leads to end-associations in M1 and those that lead to end-fusions, dicentric chromosome formation, and apoptosis during M2. One model consistent with the present results is that non-canonical telomeric sequence variants at the base of the telomeres could produce mismatches with the invading G-rich overhangs and destabilize t-loop formation. The unfolded telomeres would still have intact G-rich 3' overhangs and sufficient double-stranded telomeric sequences to provide partial masking from the DNA repair apparatus. A partial DNA damage response might initiate end-associations with another telomere that are "transient" (i.e., either do not involve actual covalent ligation of the ends, or where breakage is preferentially induced within the short stretch of telomeric repeats so that some protective sequences still remain). The implications of non-canonical sequences for pathologies involving premature replicative aging will be discussed.